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Identification of a Cytopathogenic Agent Infectious for Puppies as a Canine Herpesvirus.* (30614)

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(Introduced by J. A. Baker)
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A fatal disease of infant puppies caused by cytopathogenic organisms which initially were believed to be mycoplasma has been reported(1). Further study revealed, however, that certain tissues from puppies that died contained 2 organisms, a virus and mycoplasma. After attempts failed to relate the mycoplasma isolates to cytopathology in dog kidney cell (DKC) cultures or to the fatal illness in young puppies, it became apparent that the mycoplasma were not causally related to the disease. Moreover, cytopathic effects (CPE) in DKC cultures and typical pathologic changes in young puppies were produced consistently by inoculation with bacteriologically sterile materials that did not contain mycoplasma. A detailed clinical and pathologic description of the disease in puppies has been reported(2). A communication from Dr. L. N. Binn (Walter Reed Army Inst. for Research, Washington, D. C.) indicated that an agent (S-463) isolated from puppy tissues by Capt. R. O. Spertzel, and which is similar to our isolates(1), is viral and has characteristics of herpesviruses. Also, Dr. S. E. Stewart (Nat. Inst. Health, Bethesda, Md.) has reported to us that a herpes-like virus has been isolated from young puppies that died of a hemorrhagic disease.

This paper reports some of the properties of this newly recognized canine virus and compares characteristics of the virus with properties of the *Herpesvirus Group*.

Materials and methods. Tissue culture. Primary DKC cultures were prepared according to methods reported previously(1). For certain studies, secondary cell cultures were grown in glass rings on microscope slides according to Cairn's method(3). Tissue culture medium (TCM) consisted of medium 199 (GIBCO), 0.5% lactalbumin hydrolysate, 4% heated lamb serum, and antibiotics. Cultures used for testing effects of halogenated uridine compounds (see below) on viral growth were maintained in a similar medium, with the exception that Earle's saline solution was substituted for medium 199 in the formula.

Virus. Our prototype strain, designated F-205, was used in all experiments. The source of this strain was reported previously (1). Because both mycoplasma and a virus were present initially in our stock cultures, virus employed in these studies was prepared from stock in the following manner: Infected DKC cultures with advanced CPE were ultrasonicated for 1 minute at maximum frequency with a Biosonik probe (Bronwell) and emulsified for 5 minutes at 0°C with trifluorotrichloroethane (Genetron 113, Allied Chemical and Dye Corp.). Genetron-treated culture fluid was centrifuged at 30,000 rpm for 45 minutes in a #40 rotor of an ultracentrifuge (Spingo, Model L). Supernatant fluid portions were discarded and the pellets were

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pooled and resuspended in ice-cold distilled water to 1/40 the original volume. This suspension was placed on sucrose density gradient columns (20-80% sucrose) and centrifuged at 35,000 rpm for 3 hours in a SW39 rotor. After ultracentrifugation, 3 opaque bands appeared in the tubes. The bands were removed from the top with a bent 24 gauge needle attached to a syringe. A separate syringe and needle was used to remove each fraction. The lower band, which contained the major portion of infectious virus, was diluted in 10-fold steps in TCM and inoculated into DKC cultures and also into artificial media which supported growth of the contaminating mycoplasma(1). Mycoplasma were not isolated in artificial media or observed in stained coverslip preparations of DKC cultures inoculated with purified stock, however extensive CPE occurred in inoculated DKC cultures after approximately 16 hours. Purified virus was transferred an additional 2 times at the endpoint dilutions and stored as stock in sealed vials at −60°C. Such stock was designated F-205V. Before use, stock virus routinely was thawed and ultrasonicated in an ice-bath. Cell debris was sedimented by low speed centrifugation and the supernatant fluid was used as inoculum.

Cytopathology. Normal and infected DKC cultures were grown on coverslips in flat-bottom tubes and fixed in Carnoy's solution when CPE were evident. Coverslips were then rinsed in saline solution and stained with Giemsa or Schorr's stain (Harleco) and examined with a light microscope.

Size determinations. Size was estimated by ultrafiltration and electron microscopy. Ultrafiltration was performed with membrane filters (Scheicher & Schuell Co. or Millipore Co.) that varied in average pore diameter (APD) from 50 mμ through 650 mμ. One portion of stock virus was passed through filters that were prepared as recommended by the manufacturer. A second portion was not filtered. Virus titrations then were done in DKC cultures on the filtered and unfiltered stock. Detailed methods of electron microscopy are described in a separate paper(4). Briefly, infected DKC cultures with advanced CPE were fixed in gluteraldehyde followed by osmium tetroxide, soaked in 2% uranyl nitrate, and embedded in EPON-812 (Shell). Ultrathin sections were prepared and stained with lead hydroxide according to Karnovsky (5) and examined with a RCA EMU-3G electron microscope.

Sensitivity to lipid solvents. Test procedures for chloroform and ether sensitivity were followed that have been described by Feldman and Wang(6) and Andrewes and Horstmann(7).

Heat sensitivity. Suspensions of stock virus in TCM buffered at pH 7.6 with tris(hydroxymethyl)aminomethane (Sigma) were placed in constant temperature waterbaths at 36°C and 56°C; in a refrigerator at 4°C; and in a freezer at −20°C. At various intervals a sample was removed, placed in an ice-bath, and diluted in 10-fold steps. Each dilution was inoculated in 0.1 ml amounts into 3 DKC cultures. Inoculated cultures were observed for one week.

Stability at low pH. Stock virus was diluted 1:10 in cold TCM and adjusted to various pH values (pH 3 to 7.6) with McIlvane's citrate buffer. After standing for 30 minutes at 4°C, the suspensions were titrated in DKC cultures.

Nucleic acid type. Two methods were used: (1) inhibition tests using halogenated uridine compounds, 5-fluoro-2-deoxyuridine† (FUDR) and 5-iodo-2-deoxyuridine (IUDR); (2) autoradiography of infected cultures grown in glass rings on microscope slides and exposed to tritium-labeled methyl thymidine (New England Nuclear Corp.). Procedures for treatment of cell cultures with halogen derivatives of deoxyuridine were essentially the same as described by Hamparian *et al* (8) and Lam and Atherton(9). Autoradiographic methods have been described(10). Cultures of inoculated and uninoculated DKC were exposed to tritium-labeled thymidine for 15 minutes when CPE were just evident (approximately 16 hours after inoculation). Cultures were washed 3 times in phosphate buffered saline (PBS), pH 7.4, and fixed in Carnoy's solution. The fixed slides were then layered with a thin coat of liquid nuclear

† Courtesy of Hoffmann-LaRoche, Inc., Nutley, N. J.

emulsion (Kodak NTB-3) and placed in a sealed double container for 2 weeks. After development, inoculated and control slides were stained by the acridine orange fluorescent method and examined with a fluorescence microscope (Carl Zeiss, Inc.). Controls consisted of infectious canine hepatitis (ICH) virus, an adenovirus(11), and the Rockborn cytopathogenic strain of canine distemper virus(12), a probable myxovirus(11).

Hemagglutination. Agglutination of erythrocytes from various species by strain F-205V was tested at pH 6.5 and 7.4. Stock virus was diluted in 2-fold steps from 1:2 through 1:512 in PBS and each virus dilution was added in 0.5 ml amounts to 0.5 ml of a 0.2% suspension of washed erythrocytes. Donor species were fowl, guinea pig, horse, bovine, sheep, dog, mouse, and human-O. Tests were done at 4°C and at 22°C. Results were recorded after 4 hours and again after approximately 12 hours.

Serologic comparisons. Strain F-205V was compared with certain viruses that have been tentatively classified as herpesviruses(11). Antiserum to F-205V was prepared in young beagles from the Institute's dog colony. Two dogs were given an initial 5 ml intravenous inoculation with virus that had been ultracentrifuged and resuspended (pellet portion) to 1/10th the original volume in PBS. Four weeks later a second 5 ml intramuscular inoculation was given. The final inoculum consisted of virus, prepared as described above, suspended in an equal volume of Freund's complete adjuvant (Difco). Blood samples were collected 3 weeks after the final inoculation and the serum portions were tested for neutralizing antibody.

Antiserums to equine rhinopneumonitis (ER), bovine rhinotracheitis (RT), herpes simplex (HS), and pseudorabies (PR) viruses were prepared in rabbits (ER, RT and HS viruses) or guinea pigs (PR virus) by repeated inoculations with virus pellet suspensions from ultracentrifuged infectious tissue culture fluids. Guinea pigs were inoculated initially with suspensions of ultraviolet light-inactivated PR virus for 3 consecutive weeks before receiving a final inoculum of live virus 3 weeks later. By neutralization tests, preinoculation serums did not have inhibitory activity against the homologous virus. Postinoculation blood was collected when test serum diluted 1:10 completely neutralized at least 500 $TCID_{50}$ of homologous virus.

For neutralization tests, serums were heated at 56°C for 30 minutes and diluted in TCM in 3-fold steps from an initial 1:5 dilution. Three ml of stock virus diluted to contain approximately 100 $TCID_{50}$/0.1 ml were mixed with 3 ml of each serum dilution and allowed to stand for 2 hours at room temperature. The serum-virus mixtures were then inoculated in 1.5 ml amounts into each of 3 cell cultures from which growth medium had been removed. Dog kidney cell cultures were used for F-205V and RT tests; swine kidney cell cultures were used for ER and PR tests; and HeLa cell cultures were used for HS neutralization tests. Inoculated and uninoculated tubes were placed at 36°C in stationary racks and observed daily for one week. Although this method was not necessary to assay antibody for all of the above viruses, we could not demonstrate antibody for strain F-205V, except in low serum dilutions, by conventional neutralization tests.

Results. Viral growth and cytopathology. From one-step growth experiments (Fig. 1), virus was mostly cell-associated. Maximal virus infectivity titers occurred at the time when CPE were first evident microscopically in unstained cultures. Cytopathic effects were noted first 12 to 18 hours after inoculation with approximately 10^4 $TCID_{50}$. Unstained and stained cell cultures infected with the mycoplasma-free stock (F-205V) had essen-

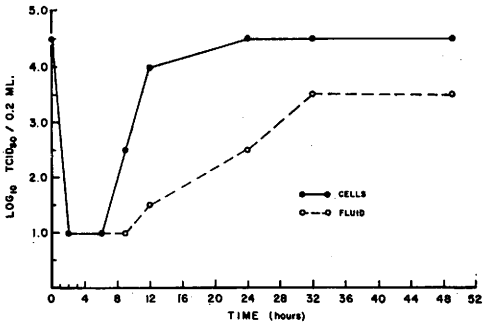

FIG. 1. One-step growth curve of strain F-205V in DK cell cultures.

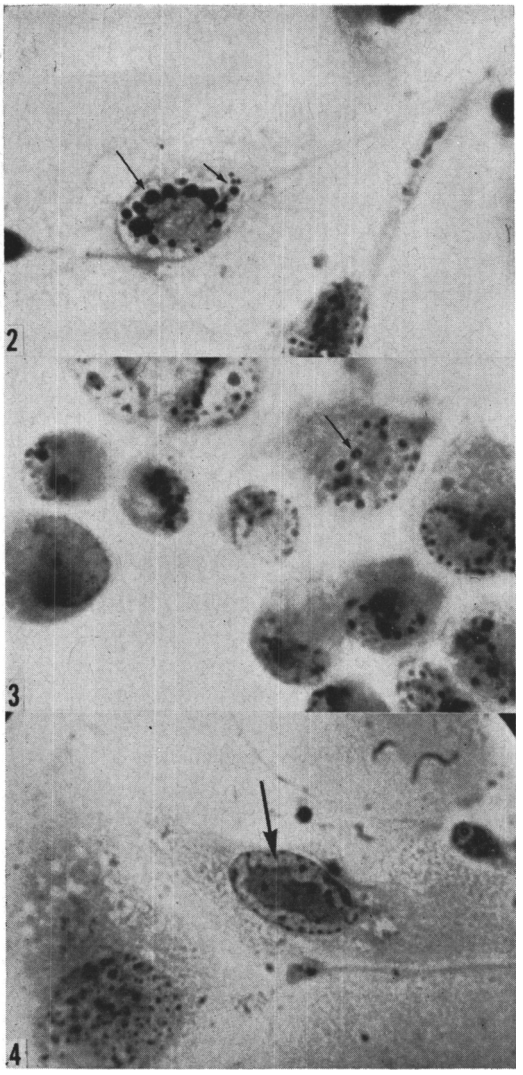

FIG. 2. Cytopathic effects produced in DKC cultures inoculated with strain F-205V. Intranuclear bodies (arrow) are characteristic. Schorr's stain. ×300.

FIG. 3. Cytologic changes in infected DKC cells. Some intranuclear bodies have faintly-staining centers (arrow). Schorr's stain. ×300.

FIG. 4. Infected DKC culture. Acidophilic intranuclear inclusion. Schorr's stain. ×300.

tially the same CPE as described in DKC cultures inoculated with mycoplasma-contaminated stock(1), with the exception that coccoid and coccobacillary bodies were not seen. Typical CPE caused by strain F-205V consisted of foci of rounded cells that detached from the glass leaving cleared plaques surrounded by degenerating cells. Most

TABLE I. Size Determination of F-205V by Ultrafiltration Through Membrane Filters.

Filter size (APD)*	Virus titer after filtration†
50	<1.0
100	<1.0
150	<1.0
300	2.8
450	2.8
650	3.5
Control (pre-filtration)	3.8

* Avg pore diameter in mμ.
† Titer expressed as $TCID_{50}$/0.1 ml inoculum.

marked changes that were observed in stained cultures occurred in nuclei. These consisted of apparent dissolution of chromatin and, characteristically, the appearance of basophilic bodies within the nucleoplasm adjacent to the nuclear membrane (Fig. 2). These bodies appeared purple or violet with Giemsa's stain; however, with Schorr's stain they were magenta. Many of these bodies appeared to have lighter-staining centers which were faintly acidophilic (Fig. 3). Although cells occasionally had faintly acidophilic, single intranuclear inclusions (Fig. 4), these were not typical. Changes noted above did not occur in uninoculated DKC cultures.

Size. Results of ultrafiltration studies are shown in Table I. Using the filter factors suggested by Black(13) for estimating the average size of virus particles, the hydrated particle size was approximately 144 mμ.

Electron microscopy of thin sections of infected cells revealed particles that were similar to herpesviruses(14). Virus particles had an electron dense central core which, in sections through the center of the virion, appeared cylindrical and distinctly helical (Fig. 5). Particles were most numerous in the nucleus and within cytoplasmic vacuoles. The cylindrical cores appeared helical and had a mean diameter of 41 mμ (range 35 to 55 mμ). Cores surrounded by a single membrane were 74 mμ (range 65 to 85 mμ), and particles with an inner membrane surrounded by an outer envelope had an average diameter of 142 mμ (range 115 to 175 mμ). Most particles appeared ovoid; however, the shapes observed in thin sections were probably compression artifacts. Particles negatively stained

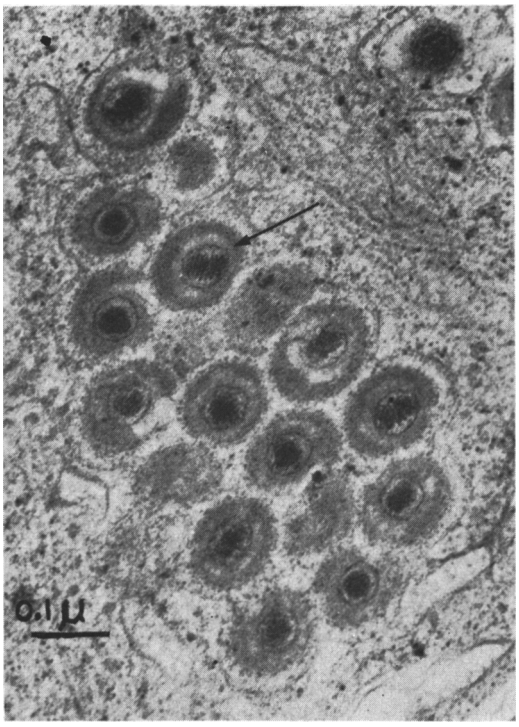

FIG. 5. Electron photomicrograph of virus particles as seen in thin section of infected DKC culture. Note helical core (arrow).

with neutral phosphotungstic acid were clearly icosohedral(4).

Sensitivity to lipid solvents. Virus infectivity was destroyed by overnight treatment with diethyl ether (20% by volume) at 4°C. Virus also was inactivated at 4°C after addition of chloroform to a final concentration of 5%, followed by shaking for 10 minutes. These treatments did not cause inactivation of ICH virus. Canine distemper virus, however, was inactivated.

Heat sensitivity. Virus suspended in TCM was rapidly inactivated at 56°C (Fig. 6). More than 99% of the original infectivity was destroyed after exposure to 56°C for 4 minutes. The half-life was approximately 5 hours at 36°C, and infectivity was not detected after 16 hours at this temperature. At refrigerator temperature (4°C), virus was stable for approximately 24 hours; more than 90% was inactivated after one week. Infectious virus could not be recovered after 3 weeks from suspensions stored at 4°C. At —20°C virus was stable for 5 days; however, infectivity declined gradually and more than 99% was inactivated after 28 days.

Stability at low pH. Virus suspended for 30 minutes at 4°C in TCM buffered at various pH values was completely inactivated below pH 4.5. At pH 6.5 through 7.6 there was no reduction in titer. Approximately 90% reduction in titer occurred at pH 5.5; and more than 99% loss of infectivity occurred at pH 4.8.

Hemagglutination. There was no agglutination of fowl, guinea pig, bovine, horse, sheep, dog, mouse, or human-O erythrocytes at pH 6.5 or 7.4. Results were the same when tests were done at 4°C and at 22°C.

Nucleic acid type. Inhibition tests that employed FUDR and IUDR incorporated into TCM at 10^{-6} M and 10^{-5} M, respectively, indicated that strain F-205V is a deoxyvirus (Table II). Consistent results were obtained when TCM that contained inhibitor was added 2 hours before virus inoculation and allowed to remain throughout the observation period of 6 days. Inhibition was not demonstrated by the indirect method(8), even though FUDR and IUDR were incorporated into TCM at 10^{-4} M. By the direct test, ICH virus was inhibited. In contrast, there was no effect of the inhibitor compounds on canine distemper virus.

Autoradiography of cultures infected with strain F-205V and exposed to tritium-labeled methyl thymidine indicated increased DNA synthesis in infected cells (Fig. 7). In infected cells, only a portion of the cells surrounding foci of degeneration appeared to be actively incorporating thymidine and these seemed to be in early stages of infection. Cells in advanced stages of degeneration did

TABLE II. Effect of FUDR and IUDR on Growth of Strain F-205V.

Virus	Log_{10} virus titer*		
	FUDR	IUDR	Control†
F-205V	2.5	1.0	4.0
Canine hepatitis	3.5	3.2	6.5
" distemper‡	3.5	3.5	3.5

* Titer per 0.1 ml inoculum.
† Final virus titer in TCM from which halogenated pyrimidines were omitted.
‡ Rockborn cytopathogenic strain.

TABLE III. Serological Comparisons of Strain F-205V and Certain Herpesviruses.

Virus	Antibody titer* Hyperimmune serum F-205V	ER	RT	HS	PR
F-205V	1:800	—†	—	—	—
Equine rhinopneumonitis (ER)	—	>1:10	—	—	—
Bovine rhinotracheitis (RT)	—	—	>1:10	—	—
Herpes simplex (HS)	ND‡	ND	ND	>1:10	ND
Pseudorabies (PR)	—	—	—	—	>1:10

* See *Methods* for test procedures.
† No neutralization of test virus at 1:5 serum dilution.
‡ ND = not done.

not appear to be incorporating thymidine, for reduced silver grains were rarely seen in the emulsion above nuclei of pyknotic and rounded cells. Grain patterns were clearly different from those in dividing cells in both inoculated and control cultures. Globular bodies similar to structures observed in nuclei of cells stained with Giemsa or Schorr's stains fluoresced green-yellow by the acridine orange fluorescent method indicating that they contained both DNA and ribonucleic acid (RNA). In cultures with advanced CPE, much of the nucleoprotein appeared to be condensed into these compact bodies which frequently had extruded from nuclei into the cytoplasm. Nuclei of many degenerated cells appeared vacuolar or empty, as though the chromatin had dissolved or had been digested.

Serologic comparisons. Infectivity of strain F-205V was not neutralized by hyperimmune serums that had homologous neutralizing antibody against ER, RT, HS, and PR viruses (Table III). It was shown previously(1) that strain F-205 was not ICH virus or distemper virus.

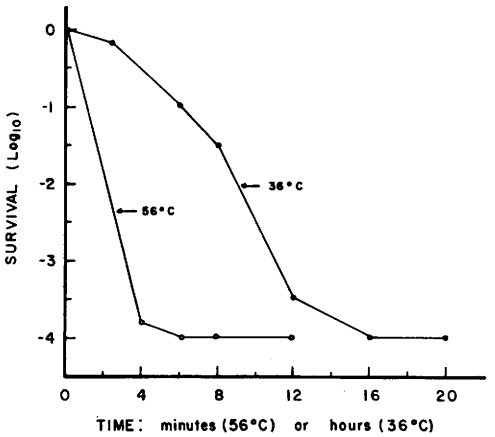

FIG. 6. Heat inactivation of strain F-205V at 56°C and 36°C.

Discussion. Our earlier report(1) of a fatal hemorrhagic disease of puppies caused by mycoplasma was incorrect as regards the causal agent; it now appears clear that the disease is viral. The mycoplasma in our early cultures were contaminants present in some of the original pathologic specimens. The typical disease has been produced with mycoplasma-free inocula(2) and communications from 2 additional laboratories(15,16) on a similar fatal disease of young puppies also indicates that the causal agents are viral. An exchange of isolates with one of the laboratories(15) indicated that strains F-205V and S-463 are culturally and serologically similar (1).

The data obtained from this study indicate that strain F-205V is a canine herpesvirus. Enveloped virus particles observed in thin sections of infected cells had an average diameter of 142 mμ with an apparently DNA-containing nucleocapsid that measured 65 to 85 mμ. Additional studies(4) have indicated that the virion is icosohedral, with the capsid composed of 162 capsomeres.

Although classical type-A intranuclear inclusions were not typical of infected cells, faintly acidophilic intranuclear inclusions occasionally were observed in cells in tissue cultures and also in necrotic areas of organs from inoculated puppies(2). A characteristic cytologic change was the presence of nucleoprotein bodies within infected cell nuclei. These bodies were seen also in the cytoplasm of some cells. Cytological changes were quite similar to the initial nuclear alterations in

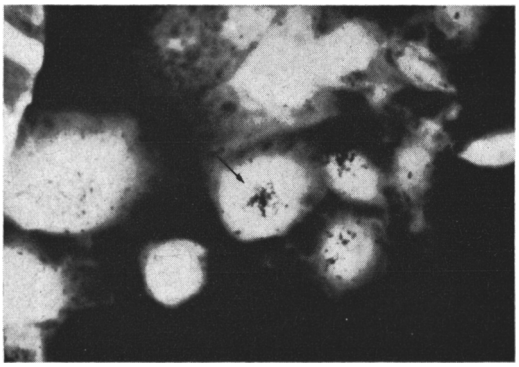

FIG. 7. Autoradiograph of dog kidney cells infected with strain F-205V. Grain patterns represent 15 min exposure to tritium-labeled methyl thymidine. Acridine orange stain. ×240.

HeLa cells caused by herpes simplex virus (17). By electron microscopy, most virus was within the nucleus, where both naked and enveloped particles were seen. Virus also occurred within cytoplasmic vacuoles. Most particles within the cytoplasm were enveloped, suggesting that the developmental cycle was initiated in the nucleus.

The virus was inactivated by certain lipid solvents and is heat labile. Virus stability was greatest above pH 6.5, and virus was inactivated below pH 4.5. Strain F-205V did not agglutinate erythrocytes from various species under different conditions of pH and temperature. By neutralization tests, strain F-205V was not related to equine rhinopneumonitis, bovine rhinotracheitis, herpes simplex, or pseudorabies viruses.

In size, morphology, apparent DNA composition, intranuclear site of development, cytopathology, and sensitivity to various physical and chemical agents, strain F-205 appears to represent a canine member of the Herpesvirus Group.

Summary. An agent (strain F-205V) isolated in dog kidney cell cultures from puppies that died of a fatal hemorrhagic disease has been identified as a cytopathogenic virus. Properties of the virus indicate that it is a canine herpesvirus.

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